Journal of the American College of Cardiology
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Published by Elsevier Inc.

Vol. 55, No. 5, 2010
ISSN 0735-1097/10/$36.00
doi:10.1016/j.jacc.2009.08.064

PRE-CLINICAL RESEARCH

Rac1-Induced Connective Tissue Growth Factor Regulates Connexin 43 and N-Cadherin Expression in Atrial Fibrillation

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Objectives We studied the signal transduction of atrial structural remodeling that contributes to the pathogenesis of atrial fibrillation (AF).

Background Fibrosis is a hallmark of arrhythmogenic structural remodeling, but the underlying molecular mechanisms are incompletely understood.

Methods We performed transcriptional profiling of left atrial myocardium from patients with AF and sinus rhythm and applied cultured primary cardiac cells and transgenic mice with overexpression of constitutively active V12Rac1 (RacET) in which AF develops at old age to characterize mediators of the signal transduction of atrial remodeling.

Results Left atrial myocardium from patients with AF showed a marked up-regulation of connective tissue growth factor (CTGF) expression compared with sinus rhythm patients. This was associated with increased fibrosis, nicotinamide adenine dinucleotide phosphate oxidase, Rac1 and RhoA activity, up-regulation of N-cadherin and connexin 43 (Cx43) expression, and increased angiotensin II tissue concentration. In neonatal rat cardiomyocytes and fibroblasts, a specific small molecule inhibitor of Rac1 or simvastatin completely prevented the angiotensin II–induced up-regulation of CTGF, Cx43, and N-cadherin expression. Transfection with small-inhibiting CTGF ribonucleic acid blocked Cx43 and N-cadherin expression. RacET mice showed up-regulation of CTGF, Cx43, and N-cadherin protein expression. Inhibition of Rac1 by oral statin treatment prevented these effects, identifying Rac1 as a key regulator of CTGF in vivo.

Conclusions The data identify CTGF as an important mediator of atrial structural remodeling during AF. Angiotensin II activates CTGF via activation of Rac1 and nicotinamide adenine dinucleotide phosphate oxidase, leading to up-regulation of Cx43, N-cadherin, and interstitial fibrosis and therefore contributing to the signal transduction of atrial structural remodeling. (J Am Coll Cardiol 2010;55:469–80) © 2010 by the American College of Cardiology Foundation

Atrial fibrillation (AF) is the most common arrhythmia. Structural remodeling is an important substrate in AF (1). Structural abnormalities including myocytic hypertrophy, central loss of sarcomeres, and interstitial fibrosis result in an increase of conduction heterogeneity, which facilitates the maintenance of AF (2). The molecular mechanisms of atrial structural remodeling are incompletely understood.

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Recent evidence shows that increased atrial oxidative stress contributes to inducing and maintaining AF (1,3–5). The left atrium (LA) of patients with AF is characterized by increased Rac1 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (4). The Rho guanosine triphosphatase (GTPase) Rac1 regulates NADPH oxidase activity and is critical for generating oxidative stress and producing cardiac left ventricular hypertrophy (6–8). No-

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Manuscript received March 10, 2009; revised manuscript received August 7, 2009, accepted August 10, 2009.

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Abbreviations and Acronyms

AF = atrial fibrillation
CTGF = connective tissue growth factor
Cx = connexin
ECG = electrocardiography
GTPase = guanosine triphosphatase
HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A
LA = left atrium
NADPH = nicotinamide adenine dinucleotide phosphate
np-Cx43 = nonphosphorylated connexin 43
qRT-PCR = quantitative real-time polymerase chain reaction
RacET = transgenic mice with cardiac-specific overexpression of a constitutively active Rac1
RNA = ribonucleic acid
siRNA = small interfering ribonucleic acid
SR = sinus rhythm
TGF = transforming growth factor
WT = wild-type

tably, treatment with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) is able to inhibit Rac1 activation by inhibiting its geranylgeranylation and membrane translocation (4,9,10). In agreement with these observations, activation of the local renin-angiotensin system in atrial tissue has been found to play an important role in atrial structural remodeling (11). Rac1 GTPase is a mediator of angiotensin II in the cardiovascular system (6,10). Angiotensin II is known to cause fibrosis in the kidney, liver, lung, and myocardium (12). Fibrosis is a hallmark of arrhythmogenic structural remodeling (2,13). There are few data regarding structural remodeling and fibrosis in human LA available. Therefore, we performed transcriptional profiling of LA myocardium from patients with AF and sinus rhythm (SR) and applied cultured primary cardiac cells and transgenic mice to characterize mediators of the signal transduction of atrial remodeling.

Methods

Cell isolation and culture. Cardiomyocytes and fibroblasts were isolated from neonatal Sprague-Dawley rat hearts (Charles River, Sulzfeld, Germany) (6). Purity of fibroblasts was confirmed by vimentin staining. After 48 h in culture, myocytes exhibited regular spontaneous contractions. Cells were used for experiments after 3 to 6 days of culture. The H9C2 embryonal rat (BD1X) heart-derived cells were obtained from American Type Culture Collection (Wesel, Germany).

Animal studies. Mice with cardiac overexpression of constitutively active (V12) Rac1 under the control of the α-myosin heavy chain promoter (RacET, provided by Prof. Mark A. Sussman, SDSU Heart Institute and Department of Biology, San Diego State University, San Diego, California) and their friend virus B-type N strain wild-type (WT) controls (14) were fed with normal chow (ssniff, Soest, Germany) or normal chow supplemented with 0.4 mg/d of commercially available rosuvastatin (Crestor, AstraZeneca, Wedel, Germany) for 10 months. The study was not funded by the pharmaceutical industry. Overall, 44% of RacET showed AF compared with 20% of RacET plus statin treatment ($p < 0.05$). None of the WT mice had AF (4). The Alb/transforming growth factor (TGF)-β1 (cys223,225ser) transgenic mice (TGF-β1-TG, provided by Stephan Rosenkranz, Klinik III für Innere Medizin, Universität Köln, Köln, Germany) were compared with their C57BL/6J WT controls (15). The study was approved by the Animal Ethics Committee of the Universität des Saarlandes and is in accord with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996).

Human left atrial tissue. Tissue samples of the left atrial appendage of patients undergoing mitral valve surgery were analyzed in 5 patients with SR and in patients with permanent AF (documented by electrocardiography [ECG] for >3 months; 5 patients per group, 4 males and 1 female, respectively). There was no difference in atrial diameter, left ventricular function, medication, and age (SR 67 ± 6 years vs. AF 63 ± 8 years, p = NS) between both groups. The patients did not receive drugs at least 12 h before surgery; no patient was treated with a statin. The study of human myocardial tissue was approved by the Ethics Committee of the Ärztekammer des Saarlandes (No. 131/00).

RIBONUCLEIC ACID (RNA) ISOLATION. The RNA was isolated following the manufacturer's instructions for total RNA isolation from fibrous tissues (Qiagen, Hilden, Germany). RNA concentration and quality were assessed using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, Delaware) and an RNA 6000 Nano LabChip on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California).

LINEAR AMPLIFICATION AND ARRAY HYBRIDIZATION. Transcriptional profiling of heart tissue of AF patients was performed with GeneChip-Human Genome-HGU 133-Plus 2.0 arrays (Affymetrix, Santa Clara, California). Two-round T7-RNA polymerase-mediated linear amplification was performed starting with 50 ng RNA (2-cycle target labeling protocol). Biotin-labeled amplified RNA size distribution was analyzed with an Agilent 2100 BioAnalyzer, and concentrations were determined using a NanoDrop ND-1000. After hybridization and washing, arrays were scanned using the GeneChip System Confocal Scanner 3000 (Affymetrix).

DEOXYRIBONUCLEIC ACID ARRAY DATA ANALYSIS. Affymetrix HGU133-Plus 2.0 arrays query the expression of 54,675 probe sets. After analysis with Gene Chip Operating software, expression raw data were transferred to GeneSpring GX version 7.3.1. (Agilent Technologies) to perform per-chip normalization for signals that were flagged in Gene Chip Operating software as present or marginal. Before statistical analysis, probe sets were filtered on the basis of their flags. Filtering was based on 80% of present/marginal signals in the Affymetrix data in at least 1 of the classes compared (control group vs. AF patients). The Cyber-T statistics program (University of California, Irvine, Califor-

nia) was used for the identification of genes differentially expressed between controls and AF patients. Application of Cyber-T results in an average ratio and a p value for each probe set. Expression data have been submitted to the National Center for Biotechnology Information gene-expression and hybridization-array data repository.

MEASUREMENT OF ANGIOTENSIN II CONCENTRATION. Angiotensin II concentration was determined by enzyme-linked immunosorbent assay using Assay Max (Assay Pro, St. Charles, Missouri).

NADPH OXIDASE ACTIVITY. NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay, as described (6).

WESTERN BLOT ANALYSIS. Immunoblotting was performed using Anti-Rac1 (Rac1, Upstate, Billerica, Massachusetts, clone 23A8), connexin 43 (Cx43 [Sigma, München, Germany]), nonphosphorylated Cx43 (np-Cx43 [Invitrogen, Karlsruhe, Germany]), connexin 40 (Cx40 [Santa Cruz Biotechnology, Santa Cruz, California]), N-cadherin (Santa Cruz Biotechnology), TGF-β1 (Santa Cruz Biotechnology), RhoA (Santa Cruz Biotechnology), and Tubulin (Santa Cruz Biotechnology), as described (6).

SMALL INTERFERING RIBONUCLEIC ACID (SIRNA) TRANSFECTION. Transfection of H9C2 with CTGF siRNA (sc-39330, Santa Cruz Biotechnology) was performed according to the manufacturer's instructions; fluorescein conjugated scrambled siRNA (sc-36869) was used as control.

RAC1 GST-PAK AND RHO GST-RHOTEKIN PULL-DOWN ASSAY. Pull-down assays were performed using agarose-labeled PAK-1 fusion protein and Rhotekin fusion protein (both Upstate), as described (4).

REAL-TIME POLYMERASE CHAIN REACTION. Total RNA was isolated from human left atrial myocardium using peqGOLD TriFast (Peqlab Biotechnology GmbH, Erlangen, Germany) and transcribed using HighCap cDNA RT Kit (Applied Biosystems, Darmstadt, Germany). Gene expression was then evaluated by quantitative real-time polymerase chain reaction (qRT-PCR [StepOne Plus, Applied Biosystems]) using Power SYBR Green (Applied Biosystems). Signals were normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase controls. No template controls were used to monitor for contaminating amplifications. The ΔCt was used for statistical analysis and $2^{-\Delta\Delta Ct}$ for data presentation. Oligonucleotide probes were as follows: human TGF-β1 forward, GAC TGC GGA TCT CTG TGT CA; human TGF-β1 reverse, GGG CAA AGG AAT AGT GCA GA; human CTGF forward, TTG GCA GGC TGA TTT CTA GG; human CTGF reverse, GGT GCA AAC ATG TAA CTT TTG G; human GAPDH forward, ATG ACA TCA AGA AGG TGG TG; human GAPDH reverse, CAT ACC AGG AAA TGA GCT TG; mouse Rac1 forward, AAC CTG CCT GCT CAT CAG TT; mouse Rac1 reverse, TTG TCC AGC TGT GTC CCA TA; mouse TGF-β1 forward, AGC CCG AAG CGG ACT ACT AT; mouse TGF-β1 reverse, TCC ACA TGT TGC TCC ACA CT; mouse CTGF forward, AGC AGC TGG GAG AAC TGT GT; mouse CTGF reverse, GCT GCT TTG GAA GGA CTC AC; mouse GAPDH forward, CCC TGC ATC CAC TGG TGC TGC; mouse GAPDH reverse, CAT TGA GAG CAA TGC CAG CCC.

HISTOLOGICAL ANALYSIS. Ten-μm cryosections were stained with 0.1% Sirius Red F3BA (Laboratory Imaging, Prague, Czech Republic). Lucia Measurement software, version 4.6, was used for quantification of interstitial fibrosis.

IMMUNOFLUORESCENCE ANALYSIS. Indirect immunofluorescence was performed on paraffin-embedded sections of the human LA applying monoclonal antibodies against α-sarcomeric actin (cardiomyocytes [Sigma-Aldrich, München, Germany]), CTGF (Santa Cruz Biotechnology), Cx43 (Sigma-Aldrich), and vimentin (fibroblasts [Dako Cytomation, Glostrup, Denmark]). Fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated (Dianova, Hamburg, Germany) anti-mouse immunoglobulin (Ig) M, anti-mouse IgG, and anti-goat IgG were used as secondary antibodies. Sections were counterstained with 4′,6 diamidino-2-phenylindole (DAPI [Calbiochem, Darmstadt, Germany]). Immunostaining with fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated anti-mouse IgM, anti-mouse IgG, and anti-goat IgG as a negative control for nonspecific staining was performed in parallel sections.

Statistical analysis. Band intensities were analyzed by densitometry. Mean, standard error of the mean (SEM), and statistical analysis were calculated by Sigma Stat software, version 2.0 (Systat Software, Erkrath, Germany). Unpaired Student *t* tests and, in case of failing the normality test, the Mann-Whitney rank-sum test for single comparison were performed. For all multiple comparisons, analysis of variance followed by Newman-Keuls post-hoc analysis for multiple comparisons was applied. Differences were considered significant at $p < 0.05$.

Results

Increased CTGF expression and fibrosis in LA of patients with AF. To identify mediators of the signal transduction of atrial remodeling during AF, expression profiling was performed by Affymetrix array. Tissue samples of the LA appendage were analyzed in 5 patients with SR and 5 patients with permanent AF without differences in atrial diameter (54 ± 2.2 mm vs. 55 ± 1.8 mm, p = NS), left ventricular function (59 ± 5.6% vs. 61 ± 5.8%), and medication. Screening of ≈25,000 genes by Affymetrix arrays showed that a total of 426 genes displayed significantly altered expression patterns ($p < 0.01$), and 122 genes displayed a more than twofold change in LA of patients with AF compared with SR. In addition to down-regulation

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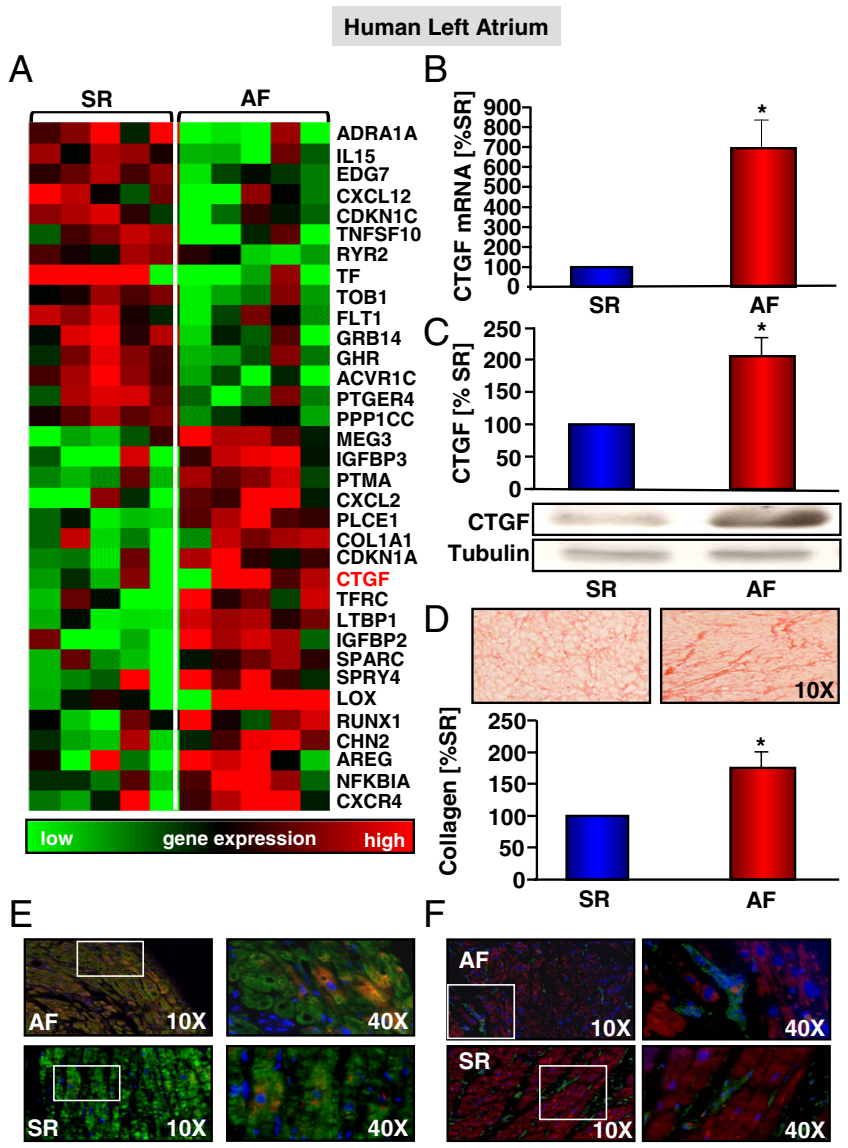

Figure 1 **Increased CTGF Expression and Fibrosis in LA of Patients With AF**

(A) Altered gene expression in patients with atrial fibrillation (AF). Ingenuity Pathway Analysis 6.0 was used to functionally classify AF-related changes in gene expression. Hierarchical clustering of left atrium (LA) tissue from patients with sinus rhythm (SR) or AF was utilized to display AF-regulated genes on proliferation-associated genes of eukaryotic cells. **(B)** Quantitative real-time polymerase chain reaction (qRT-PCR) of connective tissue growth factor (CTGF) messenger ribonucleic acid (mRNA) expression in LA of patients with SR or AF ($n = 5$). *$p < 0.05$. **(C)** Quantification and representative Western blot of CTGF protein related to tubulin expression in LA of patients with SR or AF ($n = 5$). *$p < 0.05$. **(D)** Representative sirius red staining (10-fold magnification) and quantification of interstitial fibrosis in LA of patients with SR or AF ($n = 5$). *$p < 0.05$. **(E)** Immunofluorescent imaging of CTGF protein **(red)**, the myocyte marker α-sarcomeric actin **(green)**, and cell nuclei **(blue)**, and **(F)** CTGF protein **(red)**, the fibroblast marker vimentin **(green)**, and cell nuclei **(blue).** (10- and 40-fold magnification in left atrium of patients with SR or AF.)

of genes associated with cardiac beta-adrenergic signaling (e.g., RYR2, PPP1R1A), elevated expression of genes related to remodeling (COL1A, COL1A2, COL3A1, TIMP1, CTGF) was observed (Fig. 1A). A literature search identified CTGF as a possible candidate in the pathogenesis of tissue fibrosis. Up-regulation of CTGF was confirmed by real-time polymerase chain reaction (RT-PCR [694 ± 142%, $p < 0.05$ compared with SR]) (Fig. 1B) and Western blot analysis (205 ± 28%, $p < 0.05$) (Fig. 1C). Increased expression of CTGF was associated with increased interstitial fibrosis (175 ± 25% of SR, $p < 0.05$) (Fig. 1D). Immunofluorescent staining of LA from patients with AF or SR showed a higher expression of CTGF in cardiomyocytes compared with cardiac fibroblasts (Figs. 1E and 1F).

Increased angiotensin II tissue levels, N-cadherin, and Cx43 expression in LA of patients with AF. To investigate the role of the local renin-angiotensin system in the

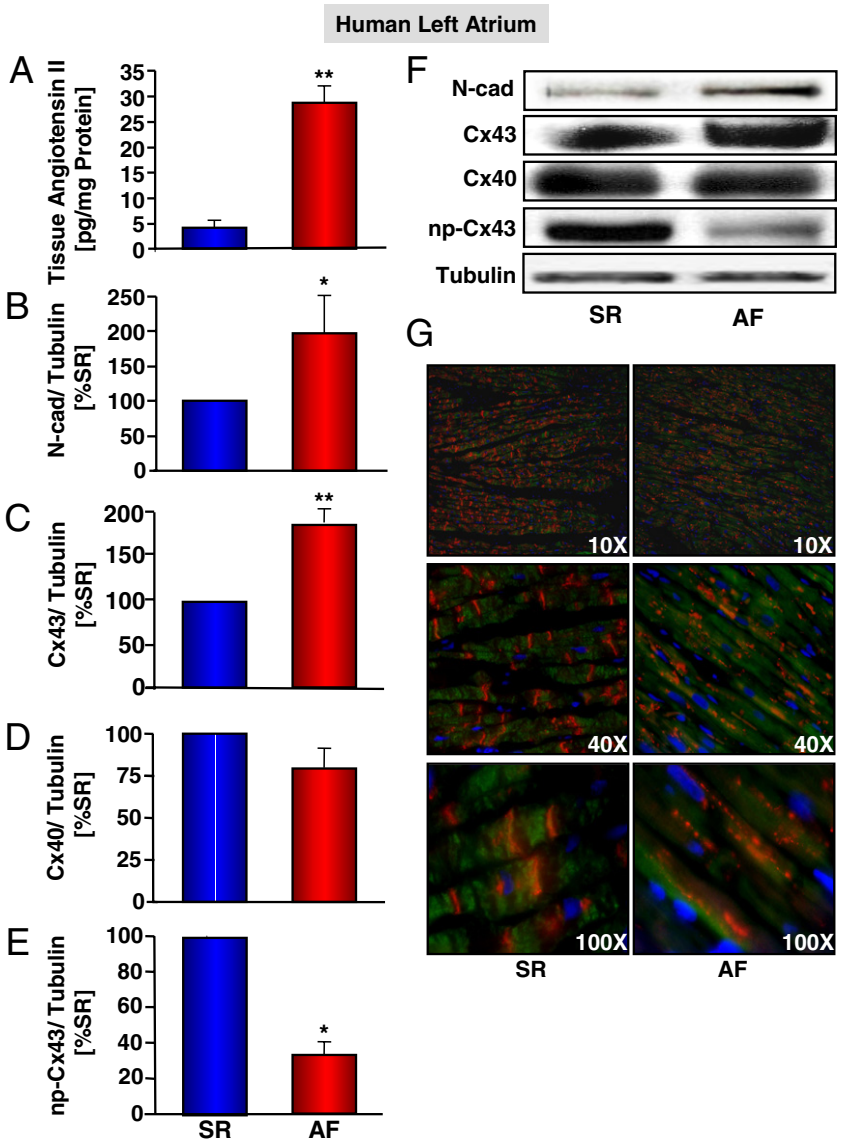

Figure 2 **Increased Angiotensin II Tissue Levels, N-Cad, and Cx43 Expression in LA of Patients With AF**

(A) Quantification of angiotensin II tissue levels, detected by enzyme-linked immunosorbent assay in LA of patients with SR or AF (n = 5). **p < 0.001). Quantification of **(B)** N-cadherin (N-cad), **(C)** connexin 43 (Cx43), **(D)** connexin 40 (Cx40), and **(E)** nonphosphorylated Cx43 (np-Cx43) protein expression related to tubulin (n = 5). *p < 0.05 and **p < 0.001 versus patients with SR. **(F)** Representative Western blots. **(G)** Immunofluorescence analysis (4′,6 diamidino-2-phenylindole) of Cx43 **(red)** localization in relation to α-sarcomeric actin **(green)** and cell nuclei **(blue)** (10-, 40-, and 100-fold magnification in LA of patients with SR compared with AF). Abbreviations as in Figure 1.

LA, we performed an enzyme-linked immunosorbent assay, which showed sevenfold increased tissue levels of angiotensin II in AF compared with SR (28 ± 3.4 pg/mg vs. 4 ± 1.4 pg/mg protein, p < 0.001) (Fig. 2A). This observation is in agreement with our previous finding of a profound up-regulation of Rac1 expression and of the superoxide-producing NADPH-oxidase in the LA of patients with AF (4). Because Rac1 plays a critical role in the activation of the cytoskeleton, the expression of N-cadherin, a transmembrane regulator of the actin cytoskeleton and calcium-dependent adhesion, was investigated (16). Western blot analysis showed a 2-fold up-regulation of N-cadherin (196 ± 54% of SR, p = 0.04) (Fig. 2B) in the LA of patients with AF. Adherens junction formation is a prerequisite for gap junction assembly (16,17). Both Cx43 and Cx40 have been shown to represent important gap junctions in the myocardium and are involved in the pathogenesis of AF (18). Western blot analysis showed up-regulation of Cx43 (188 ± 18%, p < 0.001) (Fig. 2C). In contrast, Cx40 expression was not altered (77 ± 10% of SR, p = NS) (Fig. 2D). The Cx43 function depends on phosphorylation and was therefore assessed

using an antibody that identifies np-Cx43 on serine 368. Expression of np-Cx43 was markedly down-regulated, indicating an elevation of phosphorylation in AF (33 ± 7% of SR; $p < 0.05$) (Figs. 2E and 2F). Immunostaining for Cx43 showed a lateralization and disseminated expression of Cx43 in atrial myocytes of AF patients compared with a localization at the longitudinal cell termini in SR patients (Fig. 2G).

CTGF regulates N-cadherin and Cx43 expression in neonatal cardiomyocytes and fibroblasts. To test whether Cx43 or N-cadherin is regulated by CTGF, cultured neonatal rat cardiomyocytes were treated with recombinant CTGF (1 ng/ml for 1 h), which resulted in a moderate but significant up-regulation of Cx43 protein expression (133 ± 3% of vehicle treatment, $p < 0.001$) (Fig. 3A) and an up-regulation of N-cadherin to 181 ± 17% ($p < 0.05$) (Fig. 3B). In contrast, expression of Rac1 GTPase was not affected by CTGF treatment (Fig. 3C). Because cardiomyocytes only account for ≈45% of the atrial myocardium by volume, CTGF treatments were repeated in fibroblasts, which exhibited very similar effects (Figs. 3D to 3F). To clarify the causal role of CTGF in this signal transduction,

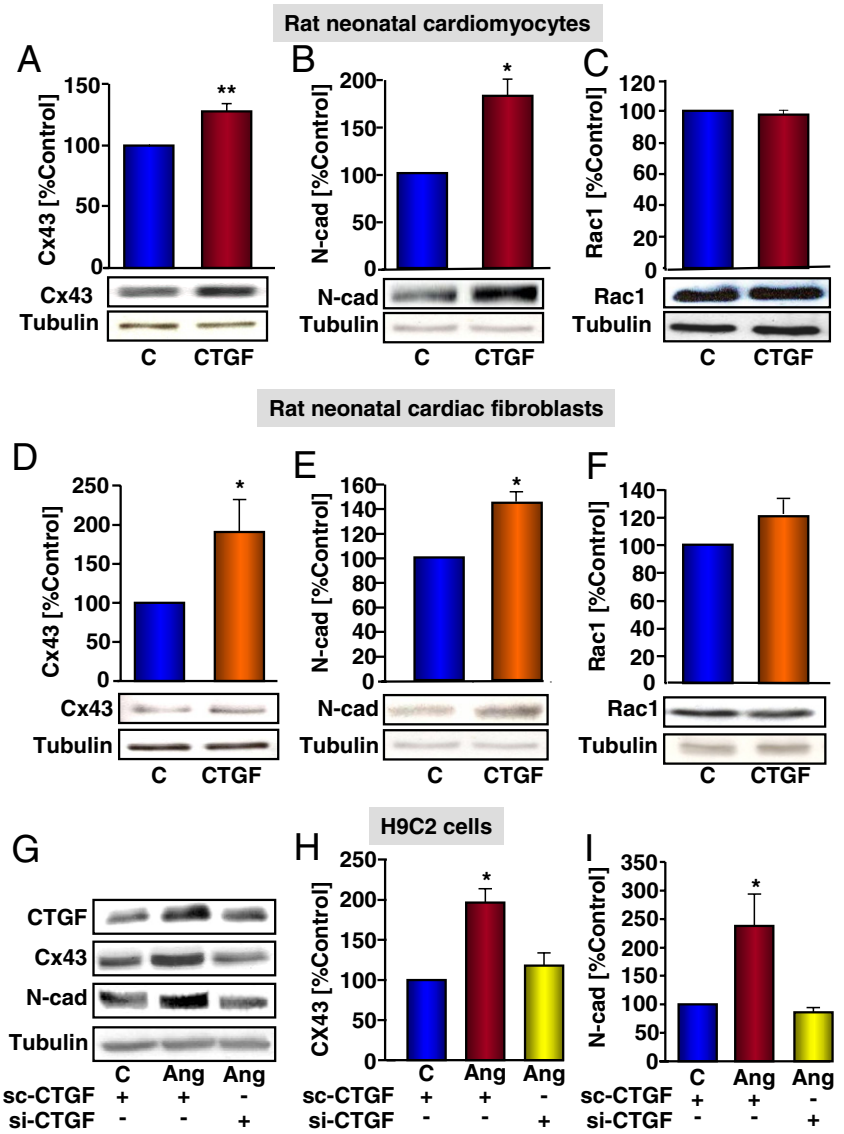

Figure 3 CTGF Regulates N-Cad and Cx43 Expression in Neonatal Cardiomyocytes and Fibroblasts

Quantification and representative Western blots showing the effects of treatment with recombinant connective tissue growth factor (CTGF) (1 ng/ml, 1 h) on **(A)** Cx43, **(B)** N-cad, and **(C)** Rac1 protein expression in neonatal rat cardiomyocytes; and on the expression of **(D)** Cx43, **(E)** N-cad, and **(F)** Rac1 in neonatal rat cardiac fibroblasts related to tubulin (n = 5). *$p < 0.05$ and **$p < 0.001$ versus control (C). **(G)** Representative Western blots showing the effect of CTGF knockdown by small interfering ribonucleic acid (siRNA) transfection (si-CTGF [sc-CTGF = scrambled ribonucleic acid]) on CTGF, Cx43, and N-cad expression in H9C2 cells. Quantification of siRNA-mediated knockdown on **(H)** Cx43 and **(I)** N-cad expression (n = 4). *$p < 0.05$. Ang = angiotensin II; other abbreviations as in Figure 2.

H9C2 cells were transfected with siRNA for CTGF. The siRNA-mediated knockdown of CTGF (Fig. 3G) prevented angiotensin-mediated up-regulation of Cx43 (196 ± 15%, $p < 0.05$) and N-cadherin (236 ± 57%, $p < 0.05$) (Figs. 3G to 3I), whereas basal Cx43 expression (83 ± 37% of control, p = NS) and basal N-cadherin expression (94 ± 54% of control, p = NS) were unaffected by CTGF-siRNA treatment.

Rac1-dependent CTGF expression in neonatal cardiomyocytes and fibroblasts. To test whether the observed activation of Rac1 GTPase in AF atria plays a causal role for the observed effects, neonatal rat cardiomyocytes and fibroblasts were stimulated with angiotensin II (1 μM for 3 h) after pre-incubation with or without the Rac1-specific small molecule inhibitor NSC23766 (100 nM, 21 h). The NSC prevents Rac1 activation by inhibition of the Rac-specific guanine nucleotide exchange factors TrioN and Tiam1 without affecting Cdc42 or RhoA activation. Western blot analysis showed up-regulation of CTGF expression after stimulation with angiotensin II to 238 ± 45%, which was completely prevented by pre-incubation with the small molecule inhibitor (53 ± 9%, $p < 0.05$) (Fig. 4A). Angiotensin II increased N-cadherin and Cx43 expression to 234% and 254% of control, respectively ($p < 0.05$) (Figs. 4B to 4D), which was completely prevented by NSC23766. These data show that Rac1 GTPase mediates the angiotensin II–induced regulation of N-cadherin and Cx43.

In addition to cholesterol lowering, inhibition of HMG-CoA reductase by statins leads to inhibition of isoprenoid synthesis. Statins have been shown to reduce Rac1 activity in cardiomyocytes by blocking Rac1 isoprenylation (6,8–10). Pre-treatment of cardiomyocytes with simvastatin (1 μM for 21 h) completely prevented the angiotensin II–induced up-regulation of CTGF ($p < 0.05$) (Figs. 4E and 4F).

These experiments were repeated in fibroblasts. Similarly to cardiomyocytes, Western blot analysis showed a significant up-regulation of CTGF expression after stimulation with angiotensin II that was completely prevented by pre-incubation with the Rac1 small molecule inhibitor (194 ± 27% angiotensin II, $p < 0.05$; 79 ± 24% angiotensin II + NSC23766, $p < 0.05$) (Fig. 5A). Treatment with angiotensin II increased N-cadherin and Cx43 expression to 138% and 180%, respectively ($p < 0.05$) (Figs. 5B to 5D), which was prevented by NSC23766, showing that Rac1 GTPase mediates the angiotensin II–induced regulation of CTGF, N-cadherin, and Cx43 in cardiac fibroblasts similar to the effects observed in myocytes.

Reduction of Rac1 activity by statin decreases CTGF, N-cadherin, and Cx43 expression in vivo. Because of the limitation of our cell culture model using ventricular cardiomyocytes and fibroblasts and to further characterize the interaction of Rac1, CTGF, and structural remodeling during AF in vivo, we studied transgenic mice with cardiac overexpression of constitutively active (V12) Rac1 under the control of the α-myosin heavy chain promoter (RacET) that spontaneously develop AF at high age (4). RacET shows a marked ~30-fold up-regulation of Rac1 activity compared with WT (4). Statin treatment (rosuvastatin 0.4 mg/day orally for 10 months; n = 10 per group) reduced Rac1 activity, superoxide release by NADPH oxidase, and the risk of AF (4). Histological analysis showed that the marked increase of Rac1 activity in RacET was associated with a significant increase of atrial interstitial fibrosis (324 ± 60% vs. WT) (Fig. 6A). Statin treatment showed a nonsignificant trend toward reducing atrial interstitial fibrosis in RacET (RacET 243 ± 40% of WT, p = 0.06) (Fig. 6A). Interestingly, RacET

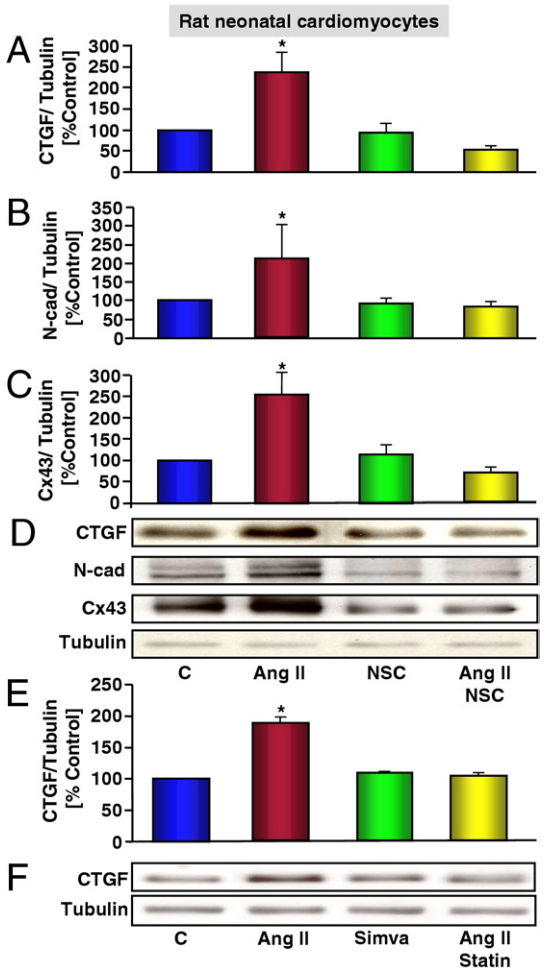

Figure 4 Rac1-Dependent CTGF Expression in Neonatal Cardiomyocytes

Effects of treatment with Ang II (1 μM, 3 h) and the specific Rac1 small molecule inhibitor NSC23766 (NSC [100 nM, 21 h]) on the expression of **(A)** CTGF, **(B)** N-cad, and **(C)** Cx43 in relation to tubulin in neonatal rat cardiomyocytes. **(D)** Representative Western blots (n = 5). *$p < 0.05$ versus C. **(E, F)** Western blot analysis of the effects of treatment with Ang II (10 μM, 3 h) and simvastatin (1 μM, 24 h) on CTGF in neonatal cardiac myocytes (n = 5). *$p < 0.05$ versus C. Abbreviations as in Figures 2 and 3.

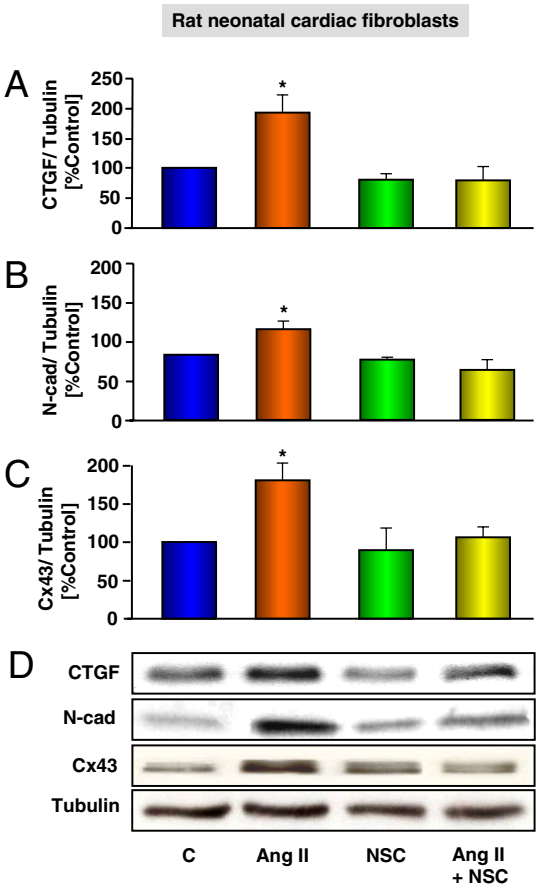

Figure 5 **Rac1-Dependent CTGF Expression in Neonatal Cardiac Fibroblasts**

Quantification of **(A)** CTGF, **(B)** N-cad, and **(C)** Cx43 protein expression in relation to tubulin in neonatal rat cardiac fibroblasts after stimulation with Ang II (1 μM, 3 h) and the Rac1 small molecule inhibitor NSC23766 (NSC [100 nM, 21 h]). **(D)** Representative Western blots (n = 5). *p < 0.05 versus C. Abbreviations as in Figures 2 and 3.

exhibited a marked increase of CTGF expression (368 ± 28% vs. WT; p < 0.001) (Fig. 6B), which was observed in RacET in AF as well as in SR. Statin treatment reduced CTGF expression to 235 ± 46% compared with WT controls. Similarly, N-cadherin (752 ± 54%, p < 0.05) (Fig. 6C) and Cx43 (317 ± 68%, p < 0.05) (Fig. 6D) were markedly increased in RacET compared with WT. Statin treatment reduced the Rac1-induced N-cadherin expression to 384 ± 21% (p < 0.05) (Fig. 6C) and reduced Cx43 expression below WT levels (84 ± 8%, p < 0.05) (Fig. 6D). Similar to the human left atria, Cx40 expression was not changed in RacET compared with WT (Fig. 6E).

TGF-β1 activates Rac1 and CTGF. The transcriptional profiling (Fig. 1A) shows that latent TGF-β1 binding protein 1, the activator of latent form of TGF-β1, is up-regulated in the LA of AF patients, which was confirmed by qRT-PCR (386 ± 84% vs. SR, p < 0.05) (Fig. 7A). TGF-β is the canonical mediator of CTGF induction (19). Expression of TGF-β1 was unchanged in RacET mice (80 ± 14% vs. WT, p = NS) (Fig. 7B). Mice overexpressing TGF-β1 were characterized by increased CTGF expression (353 ± 15% vs. WT, p < 0.05) (Fig. 7C) and Rac1 expression (214 ± 32% vs. WT, p < 0.05) (Fig. 7D). These data suggest that TGF-β1 is upstream of Rac1 and CTGF in this signal transduction.

Cross-talk between Rac1 and RhoA GTPases. Western analysis and Rhotekin-GST pull-down assay revealed a marked up-regulation of RhoA protein expression as well as activity in the LA of patients with AF (189 ± 18% vs. SR and 388 ± 75% vs. SR, respectively; p < 0.05) (Figs. 7E and 7F). RacET showed increased RhoA activity,

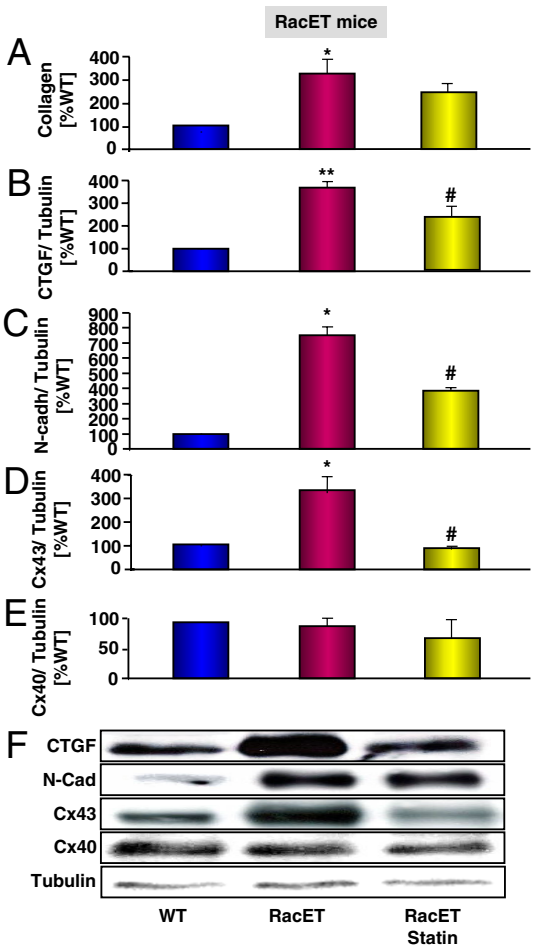

Figure 6 **Reduction of Rac1 Activity by Statin Decreases CTGF, N-Cad, and Cx43 Expression In Vivo**

Transgenic mice with cardiac overexpression of constitutively active (V12) Rac1 under the control of the α-myosin heavy chain (MHC) promoter (RacET) and RacET10 treated with rosuvastatin 0.4 mg/d orally for 10 months (RacET+Statin) compared with corresponding wild-type (WT) controls. **(A)** Quantification of atrial interstitial fibrosis by sirius red staining (n = 10). *p < 0.05 versus WT. Quantification of **(B)** CTGF, **(C)** N-cad, **(D)** Cx43, and **(E)** Cx40 protein expression related to tubulin. **(F)** Representative Western blots (n = 10 per group). *p < 0.05 versus WT. **p < 0.001 versus WT. #p < 0.05 versus RacET. Abbreviations as in Figures 2 and 3.

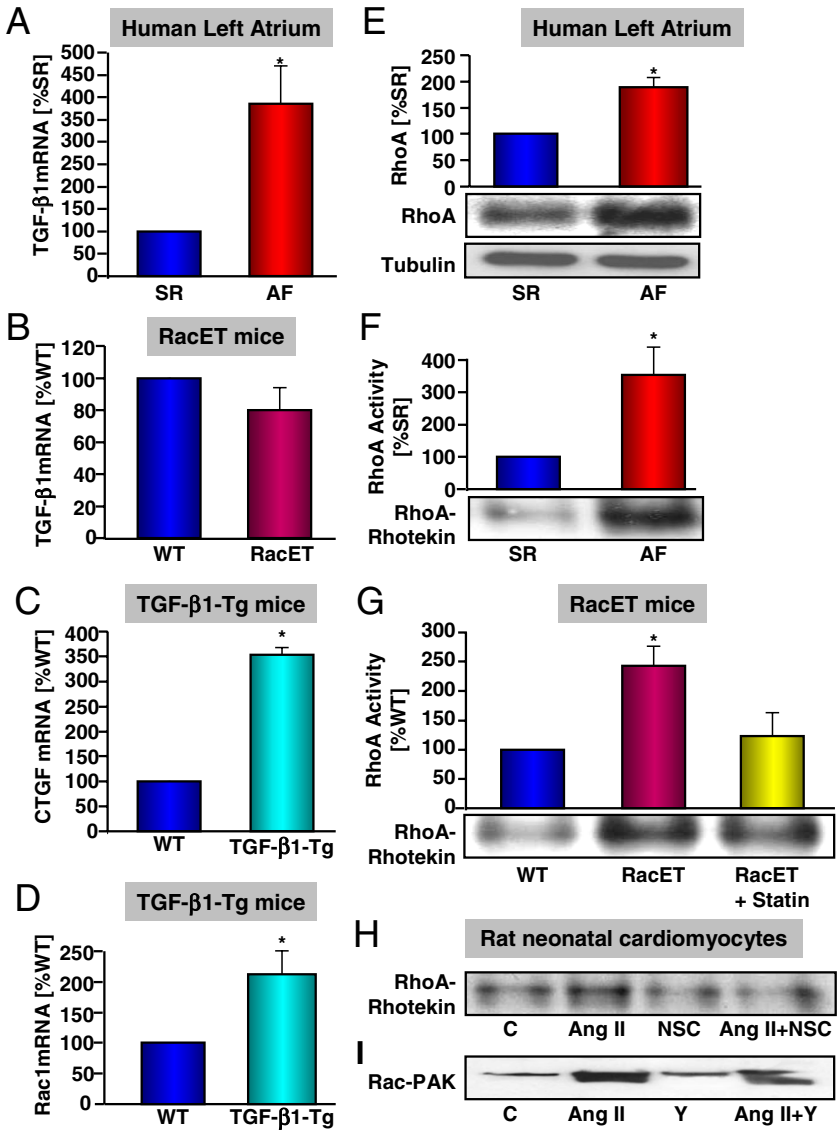

Figure 7 TGF-β1 Activates Rac1 and CTGF/Cross-Talk Between Rac1 and RhoA Guanosine Triphosphatases

qRT-PCR of transforming growth factor (TGF)-β1 messenger ribonucleic acid (mRNA) expression in **(A)** LA of patients with SR or AF ($n = 5$; $*p < 0.05$), and **(B)** RacET mice compared with wild-type (WT) controls ($n = 5$; $p = NS$). qRT-PCR of **(C)** CTGF and **(D)** Rac1 mRNA expression in TGF-β1 (cys223,225ser) overexpressing transgenic mice (TGF-β1-TG) compared with WT ($n = 5$). $*p < 0.05$. **(E)** Quantification and representative Western blot of RhoA protein expression related to tubulin, and **(F)** quantification and representative Rhotekin pull-down of RhoA activity in left atrium of patients with SR or AF ($n = 5$). $*p < 0.05$. Quantification and representative Rhotekin pull-down of RhoA activity in **(G)** RacET and RacET10 treated with rosuvastatin 0.4 mg/d orally for 10 months (RacET+statin) compared with WT controls ($n = 5$). $*p < 0.05$. **(H)** Representative Rhotekin pull-down of RhoA activity and in neonatal rat cardiomyocytes after treatment with angiotensin II (Ang II [1 μM, 3 h]) and the specific Rac1 small molecule inhibitor NSC23766 (NSC [100 nM, 21 h]). **(I)** Representative Rac1-PAK pull-down assay in neonatal rat cardiomyocytes after treatment with Ang II (1 μM, 3 h) and the selective inhibitor of Rho-associated protein kinases Y 27632 (Y [10 μM, 21 h]). Abbreviations as in Figure 1.

which was prevented by statin treatment (243 ± 32% vs. WT, $p < 0.05$) (Fig. 7G). Inhibition of Rac1 with NSC23766 (100 nM for 21 h) in neonatal cardiomyocytes reduced angiotensin II–induced RhoA activity (180 ± 9% vs. 127 ± 7% of control) (Fig. 7H). Treatment with the inhibitor of Rho-associated protein kinases Y27632 (10 μM for 21 h) reduced Rac1-activity, to a smaller extent, however, compared with the inhibition of angiotensin II–induced RhoA activity by NSC (Fig. 7I). Taken together, the data support a cross-talk between the small GTPases Rac1 and RhoA (20) in the LA myocardium and during the pathogenesis of AF.

Discussion

The data identify CTGF as an important mediator during AF in humans (Fig. 8). The study shows that angiotensin II activates CTGF via activation of the small G protein Rac1

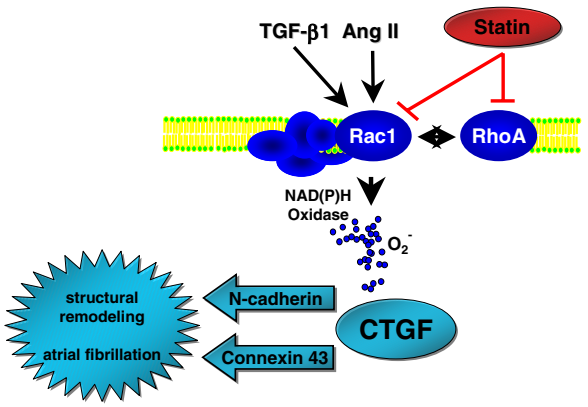

Figure 8 **Rac1-Induced CTGF Regulates Connexin 43 and N-Cadherin Expression in AF**

Activation of connective tissue growth factor (CTGF) via Rac1, leading to up-regulation of connexin 43, N-cadherin, and interstitial fibrosis, contributing to the signal transduction of atrial structural remodeling. Ang II = angiotensin II; NAD(P)H = nicotinamide adenine dinucleotide phosphate oxidase; TGF = transforming growth factor.

GTPase and NADPH oxidase activity, which contribute to the signal transduction of structural remodeling in left human atria, leading to up-regulation of Cx43, N-cadherin, and interstitial fibrosis.

Structural remodeling and contractile dysfunction of the LA play a fundamental role in the pathology of AF (2). Reactive oxygen species produced upon angiotensin II–induced activation of the Rac1-dependent NADPH oxidase contribute to atrial remodeling (4,6,8,21,22). Here, Affymetrix analysis revealed that the atria of patients with AF are characterized by up-regulation of CTGF expression. CTGF is a member of the Cyr61 (cysteine rich protein 61), CTGF (connective tissue growth factor), and NOV (nephroblastoma overexpressed gene) family of cysteine-rich secreted proteins that participate in the proliferation, migration, adhesion, and differentiation of fibroblasts. CTGF plays a role in the extracellular matrix remodeling that occurs during physiological processes such as embryogenesis, implantation, and wound healing (23,24). Increased expression of CTGF has been found in human atherosclerotic and myocardial lesions as well as in the aorta of angiotensin II–infused rats (19,25,26). Our data show that the LA myocardium of AF patients is characterized by increased angiotensin II tissue concentrations, up-regulation of CTGF, and interstitial fibrosis compared with LA from SR patients of similar size.

Experiments with neonatal cardiomyocytes and fibroblasts confirmed the up-regulation of CTGF by angiotensin II, an effect that is completely inhibited by a Rac1 specific small molecule inhibitor. Similarly, statin treatment that inhibits Rac1 activity is able to prevent the angiotensin II–induced CTGF expression. To further assess the role of myocardial Rac1 for CTGF expression, transgenic mice, overexpressing Rac1 with highly increased NADPH oxidase activity, were studied and show a marked elevation of CTGF. Importantly, AF develops in these animals at older age (4), but increased CTGF expression is observed in SR before the AF develops, suggesting a contribution of CTGF to the development of AF. Interestingly, in human lens epithelial cell line B3, reactive oxygen species increase CTGF mRNA expression, probably via activation of janus-kinase-2/-3 (27). Taken together, these data suggest that Rac1 induces CTGF expression via NADPH oxidase–generated reactive oxygen species.

The myocardial effects of angiotensin II are not limited to cardiomyocytes but are also present in fibroblasts. Indeed, angiotensin II has been shown to induce extracellular matrix protein synthesis and secretion in cardiac fibroblast. Cardiomyocytes probably do not directly synthesize collagen but influence structural remodeling through interactions with neighboring fibroblasts (28). In addition to profibrotic actions, mechanical stretch of fibroblasts may modulate myocyte electrical activity by interfering with the intercellular transfer of electrical signals.

Gap junctions formed by connexins maintain intercellular ion conduction as well as metabolic coupling. Alterations in ventricular expression and function of the major cardiac connexin, Cx43, correlate with proarrhythmic conduction slowing and connexin disorganization by redistribution to lateral cell borders, and altered phosphorylation of connexins is associated with fibrosis (29–31). Adherens junctions are responsible for mechanical coupling between myocytes (for review, see Boengler et al. [18]). In cardiomyocytes, N-cadherin is 1 of the major transmembrane components of adherins junctions. The assembly of N-cadherin precedes the accumulation of Cx43 in cultured adult myocytes (17), and gap junction formation is perturbed in N-cadherin-null myocytes (32). These findings suggest that cadherin transduces signals that control the localization of Cx43. The Rac1 GTPase has been observed to be affected by structural changes of cell to cell junctions (33–35). We therefore investigated the expression of N-cadherin and Cx43 in our 3 models. The LA of patients with AF is characterized by up-regulation of N-cadherin as well as by Cx43 expression in cardiomyocytes and fibroblasts that is prevented by NSC 23766 or siRNA-mediated knockdown of CTGF. Importantly, stimulation with recombinant CTGF leads to increased expression of N-cadherin and Cx43. Growth factors have been shown to alter N-cadherin expression during changes of cellular phenotypes, for example, during epithelial-mesenchymal transition of PMC42-LA cells treated with endothelial growth factor; however, the detailed mechanisms of the CTGF-induced changes have to be addressed in further studies (36). Taken together, the data identify CTGF as a mediator of angiotensin II–induced regulation of junction proteins during atrial fibrillation.

Angiotensin II is not the only upstream mediator of CTGF; the expression profiling and confirmation assays

show up-regulation of TGF-β1 in the LA of patients with AF. Mice overexpressing TGF-β1 are characterized by increased CTGF and up-regulation of Rac1. In contrast, RacET mice showed no significant alteration of TGF-β1 expression. Further cell culture experiments showed that stimulation with TGF-β1 triggers GTP binding to Rac1 within minutes (37), suggesting that TGF-β1 activates Rac1 and CTGF in this signal transduction. These data support the importance of CTGF in the signaling of atrial fibrosis. Further studies are needed to clarify the mechanism of TGF-β1 regulation in patients with AF and the interaction of TGF-β1 with Rac1 (37).

Conclusions

In addition to Rac1, the LA of AF patients is characterized by up-regulation of RhoA expression and activity. In agreement with the literature, our experiments show cross-talk between Rac1 and RhoA GTPases (20). In addition to contributing to the Rac1 signaling, RhoA may exert Rac1-independent effects during AF that have to be studied further. Both Rac1 and RhoA are inhibited by HMG-CoA reductase inhibitors (statins), which reduce isoprenylation-dependent small G-protein function (6,8,10). Indeed, statin treatment reduced the expression of CTGF and of Cx43 and N-cadherin in cell culture, as well as in the RacET mice. These data are in agreement with the reduction of AF in the RacET mice by statin treatment (4) and with emerging clinical evidence suggesting preventive effects of statins for patients at risk for AF (5). Therefore, we believe that further characterization of the signal transduction of the pathogenesis of AF has the potential to identify additional targets for the prevention of AF.

Acknowledgments

The authors thank Simone Jäger and Ellen Becker for their excellent technical assistance.

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Key Words: atrial fibrillation ■ Rac1 ■ CTGF ■ oxidative stress ■ connexin 43.